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(54) Method and apparatus for determination of an analyte and method of calibrating such apparatus.

(57) A method and apparatus are described for the determination of an analyte in an aqueous liquid which eliminate the biasing effect of unknown interferents which are either preformed or formed *in situ* during the determination. The method comprises physically contacting a sample of the liquid with an interactive composition for the analyte, measuring the spectrophotometric responses generated by such contact at a primary wavelength λ_1 and one or more secondary wavelengths λ_2, λ_3, ...λ_n, and determining analyte concentration or activity using the equation:

$$C = a_0+a_1[A_1+\alpha_1A_2+...+\alpha_n - 1A_n]$$

wherein C is analyte concentration or activity, A_1, A_2, ...A_n are the spectrophotometric responses observed at λ_1, λ_2, ...λ_n, respectively, and a_0, a_1, and α_1, α_2, ...α_{n-1} are constants determined according to a calibrating method. Such calibrating method is an empirical means for determining and recording in a chemical analyzer the a_0,a_1, and α_1,α_2, ...α_{n-1} constants essential for making the analyte determination.

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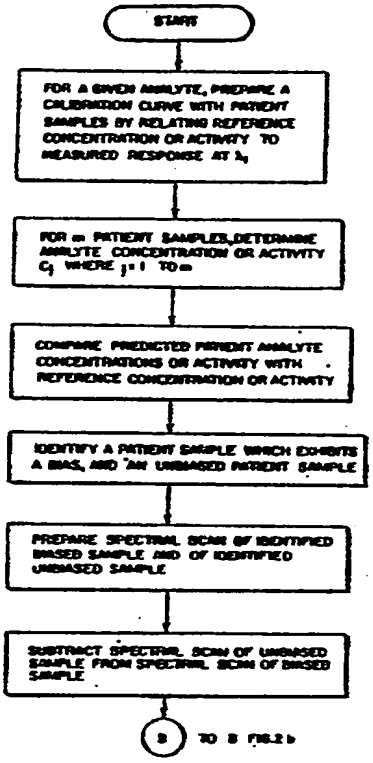

START
FOR A GIVEN ANALYTE, PREPARE A CALIBRATION CURVE WITH PATIENT SAMPLES BY RELATING REFERENCE CONCENTRATION OR ACTIVITY TO MEASURED RESPONSE AT λ_1
FOR m PATIENT SAMPLES, DETERMINE ANALYTE CONCENTRATION OR ACTIVITY C_j WHERE j = 1 TO m
COMPARE PREDICTED PATIENT ANALYTE CONCENTRATIONS OR ACTIVITY WITH REFERENCE CONCENTRATION OR ACTIVITY
IDENTIFY A PATIENT SAMPLE WHICH EXHIBITS A BIAS, AND AN UNBIASED PATIENT SAMPLE
PREPARE SPECTRAL SCAN OF IDENTIFIED BIASED SAMPLE AND OF IDENTIFIED UNBIASED SAMPLE
SUBTRACT SPECTRAL SCAN OF UNBIASED SAMPLE FROM SPECTRAL SCAN OF BIASED SAMPLE
B
TO B FIG.2b

FIG.2a

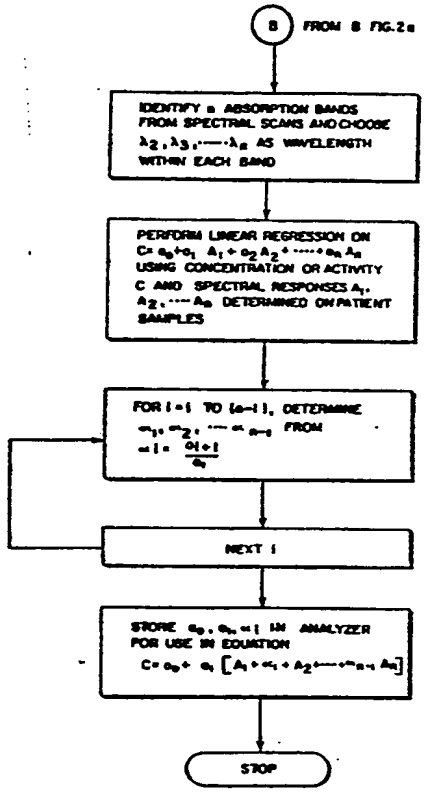

B
FROM B FIG.2a
IDENTIFY n ABSORPTION BANDS FROM SPECTRAL SCANS AND CHOOSE $\lambda_2, \lambda_3, \cdots \lambda_n$ AS WAVELENGTH WITHIN EACH BAND
PERFORM LINEAR REGRESSION ON $C = a_0 + a_1 A_1 + a_2 A_2 + \cdots + a_n A_n$ USING CONCENTRATION OR ACTIVITY C AND SPECTRAL RESPONSES $A_1, A_2, \cdots A_n$ DETERMINED ON PATIENT SAMPLES
FOR I = 1 TO (n-1), DETERMINE $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ FROM $\alpha_I = \frac{a_{I+1}}{a_1}$
NEXT I
STORE a_0, a_n, α_I IN ANALYZER FOR USE IN EQUATION $C = a_0 + a_1 [A_1 + \alpha_1 + A_2 \leftarrow \alpha_{n-1} A_n]$
STOP

FIG.2b

METHOD AND APPARATUS FOR DETERMINATION OF AN ANALYTE AND METHOD OF CALIBRATING SUCH APPARATUS

The present invention relates to a method and apparatus for determining an analyte in an aqueous liquid, such as a biological fluid. In particular it relates to a clinical chemistry analyzer and method of using same to determine analytes, such as bilirubin, in human sera. This invention also relates to a method for calibrating an apparatus, such as a chemical analyzer, to provide a means for making analyte determinations which are not biased by undefined interferents.

In order to provide desired preventative or diagnostic health care, a physician must often determine the level of various analytes in a patient's blood, urine or other body fluids. For example, the level of glucose is often important in the diagnosis and subsequent treatment of diabetes. The level of hemoglobin in the blood is often important for effective diagnosis and treatment of anemia or other related blood abnormalities.

Another important analyte which physicians often monitor is bilirubin. Bilirubin is a degradation product of hemoglobin. Approximately 200 to 230 mg of bilirubin and its derivatives are formed each day in the normal human adult. As part of normal human metabolic processes, the major portion of this daily bilirubin production is excreted or degraded into other derivatives.

Excessive amounts of bilirubin occur within the human body through overproduction of bilirubin as in the case of excessive hemolysis or by retention of bilirubin due, for example, to liver failure. The result of excessive bilirubin within the human body is jaundice. Jaundice is characterized by markedly

elevated serum bilirubin levels, for example, 10 mg of bilirubin per dl of serum or higher compared with the normal adult range of 0.1 to about 1 mg of bilirubin per dl of serum. There is increasing evidence that excessive amounts of bilirubin in the blood lead to an undesirable increase in bilirubin concentration within body cells which interferes with various cellular processes. Given this background, the clinical diagnostic significance of bilirubin, in tests for liver and other related organ functions, is self evident.

Perhaps the most widely used assay for bilirubin has been the diazo method. In this method, a sample of liquid suspected of containing bilirubin is contacted with a reagent composition which includes a diazonium salt. The diazonium salt reacts with bilirubin to form two azobilirubin fragments. The azobilirubin has an extinction coefficient which is higher than that of bilirubin itself and is easily detectable.

Many diazonium salts have been suggested for use in the diazo method for determining bilirubin. For example, certain 2,4- and 2,5-phenyldiazonium salts (e.g. 2,4- and 2,5-dichlorophenyldiazonium salts) and diazotized sulfanilamide have been used for the detection of bilirubin in serum and urine. However, methods using these diazonium salts are known to be relatively insensitive. Further, some of these diazonium salts, when dry, are explosively unstable, i.e. subject to shock induced decomposition. Thus, handling of these compounds in bilirubin assays, and particularly dry assays, is quite hazardous.

Certain substituted sulfanilamide and carbonamide diazonium salts which are less prone to shock induced decomposition have been found useful in bilirubin assays. These salts and assays are described in U.S. Patent 4,468,467 (issued August 28, 1984 to Babb et al). Those salts and assays represent a significant improvement in the clinical chemistry art, overcoming the shortcomings of previously-known bilirubin assays.

Many substances in biological fluids, both foreign and native substances, cause serious interferences in the quantitative analyses of analytes, e.g. bilirubin. Notwithstanding the significant improvement provided by the invention of U.S. Patent 4,468,467 noted above, current bilirubin assays still suffer from a significant problem. For example, with a small percentage of patient serum samples, e.g. those obtained from hemodialysis or other renal-defective patients, interferences can often be influential in the end result, detracting from assay accuracy.

Known procedures for eliminating interferences in assays include sample pretreatment, sample blanking and polychromatic (i.e. multiple wavelength) analyses. Each of these procedures, however, has its disadvantages. Sample pretreatment is a tedious and imprecise operation and is not readily adaptable to dry chemistry assays. Sample blanking doubles the effort, reagent amount and cost of each assay while being ineffective with regard to interferents formed in situ during the assay. The known polychromatic analysis requires pure standards and knowledge of the exact molecular identity or concentrations of predetermined interferents. See, e.g. Hahn et al, Clin. Chem., 25(6), pp. 951-959 (1979). This technique would not be useful where the interferent is unknown and cannot be determined prior to the assay.

None of these known procedures has proved effective for eliminating the observed interferences in bilirubin assays where neither the identity of the interferent nor its concentration (which can vary from sample to sample) is known.

The present invention provides a means for overcoming the problem of undefined interferents in the determination of an analyte, such as total bilirubin. This problem is solved with a method for calibrating a chemical analyzer useful in the determination of an analyte in an aqueous liquid. Such an analyzer comprises a) spectrophotometric means for detecting "n" spectrophotometric responses $A_1, A_2, \cdots A_n$ resulting when a sample of the liquid is contacted with an interactive composition for the analyte, and b) means for calculating the concentration or activity C of the analyte in said sample using the equation (I):

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n]$$

wherein $a_0, a_1, \alpha_1, \alpha_2, \cdots \alpha_{n-1}$ are defined hereinafter. This calibrating method is characterized by the steps of:

A. from a multiplicity of patient test samples of unknown analyte concentration or activity, identifying first and second patient test samples having substantially the same analyte concentration or activity, the first sample exhibiting a significant bias in analyte concentration or activity and the second sample exhibiting no significant bias in analyte concentration or activity measured at a primary wavelength λ_1,

B. making a spectral absorption scan of each of the samples identified in step A,

C. identifying absorption bands from the spectral scans where differences in absorbance between said scans can be observed, and selecting at least one secondary wavelength from the group of secondary wavelengths $\lambda_2, \lambda_3, \cdots \lambda_n$ representative of the absorption bands of both said first and second patient samples, respectively, wherein n represents the number of absorption bands,

D. using a multiplicity of patient test samples of known analyte concentration or activity, determining a linear regression line and its intercept and slopes using the equation (II):

$$C = a_0 + a_1A_1 + a_2A_2 + \cdots + a_nA_n$$

wherein C is analyte concentration or activity; a_0 is the intercept of the line, $A_1, A_2, \cdots A_n$ are the spectrophotometric responses measured at $\lambda_1, \lambda_2, \cdots \lambda_n$ respectively, and $a_1, a_2, \cdots a_n$ are the slopes of the line relating the spectrophotometric responses at $\lambda_1, \lambda_2, \cdots \lambda_n$, respectively, to the analyte concentration or activity,

E. using the results of step D to determine constants $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ for equation (I) above using the equation (III):

$$\alpha_i = \frac{a_{i+1}}{a_1} \text{ wherein } i = 1 \text{ to } (n-1), \text{ and}$$

F. recording in the analyzer the values of constants a_0, a_1, and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ for use in equation I above.

This invention also provides a method for the determination of an analyte in an aqueous liquid. Such method comprises physically contacting a sample of the liquid with an interactive composition for the analyte to generate a spectrophotometric response and measuring the spectrophotometric response. This method is characterized by measuring the spectrophotometric responses $A_1, A_2, \cdots An$ resulting

from such contact at, respectively, a primary wavelength λ_1 and at n secondary wavelengths selected from secondary wavelengths $\lambda_2, \lambda_3, \cdots \lambda_n$ determined according to the calibration method described above, and determining the concentration or activity C of the analyte using the equation (I):

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n]$$

wherein the constants a_0, a_1 and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ are determined according to the calibration method described above, for as many n secondary wavelengths as are used.

A chemical analyzer useful for the determination of an analyte in an aqueous liquid in contact with an interactive composition for the analyte comprises:

means for measuring the spectrophotometric responses $A_1, A_2, \cdots A_n$ at, respectively, a primary wavelength λ_1 and at n secondary wavelengths selected from the secondary wavelengths $\lambda_2, \lambda_3, \cdots \lambda_n$, and

means for determining the concentration or activity C of the analyte using the equation (I):

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n]$$

wherein the constants a_0, a_1 and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ are determined according to the calibration method described above, for as many n secondary wavelengths as are used.

This invention is practiced with an empirical calibration procedure whereby a chemical analyzer is adapted to automatically correct the assay results for any bias caused by the undefined interferent. Once the analyzer is calibrated, the assay can be performed to obtain accurate results for a population of test samples some of which may contain or be disposed to form an interferent and some of which may not. This advantage is particularly expedient when the unknown interferent is formed <u>in situ</u>, i.e. during the assay, and hence cannot be predetermined.

The present invention is particularly useful for providing a means whereby accurate total bilirubin assays can be made with an unrestricted population of serum samples. Hence, it is inconsequential to the accuracy of the assay if some of the serum samples are obtained from patients who are undergoing hemodialysis or have severe renal disorders which may otherwise produce incorrect results. Such serum samples are known as uremic serum samples.

The present invention will now be described by way of example with reference to the accompanying drawings in which:-

FIG. 1 is a schematic illustration of computing means for a chemical analyzer of the present invention and its interaction with a photodetector of the analyzer; and

FIG. 2 is a logic flow chart for the programming of the analyzer computing means.

In this specification the following are trade marks: "EKTACHEM", "Estane", "Triton", "Surfactant 10G".

The present invention is useful for measuring an analyte, such as total bilirubin, hemoglobin, glucose, uric acid, metal ions and other substances in an aqueous liquid, such as a biological liquid obtained from an animal or human. For example, the analyte can be determined in diluted or undiluted serum, plasma, whole blood, urine, cerebral spinal fluid and other body fluids with this method. It is particularly advantageous to determine total bilirubin in uremic serum with this invention.

An analyte is determined by the present invention by first physically contacting a specimen sample suspected of containing the analyte with an interactive composition for that analyte. In other words, the analyte is subjected to a composition which interacts with it in such a manner as to provide a detectable spectrophotometric response of some kind, e.g. an increase or decrease in a detectable

dye which can be detected by a suitable spectrophotometric detector, or to provide a product which of itself is not detectable, but which can further react to provide a detectable response. A detectable dye can be provided either by interaction with a dye-providing material, or by dye release from a preformed dye. The term "interaction" is meant to refer to chemical activity, catalytic activity as in the formation of an enzyme-substrate complex, immunogenic activity as in an antigen-antibody reaction, and any other form of electrical, chemical or physical interaction that can release, produce or otherwise provide a detectable response which is directly or indirectly indicative of the presence or concentration of a particular analyte. More details regarding such interactions are given, for example, in U.S. Patent 3,992,158 (issued November 16, 1976 to Przybylowicz et al).

In one embodiment, this invention can be used to determine hemoglobin and to avoid the potential interferences which can cause inaccurate hemoglobin determinations in, e.g. lipemic samples. In such determinations, hemoglobin is converted to a colorimetrically detectable species using a suitable interactive composition for hemoglobin, e.g. the conventional Drabkin's reagents (i.e. ferricyanate and cyanate).

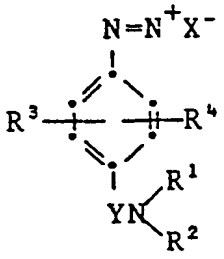
In assays for specific metallic ions practiced according to this invention, the interactive composition for a specific ion can be a chelating compound or moiety which will react or complex with that metal ion to provide a colorimetrically detectable species. In some instances, these chelating materials may be interfered with by other metal ions thereby causing premature or insufficient reaction or complexation with the desired ion. This invention can be used to reduce the effect of such interferences. In other instances, the interference

by a colored organic species with metal chelation can be reduced, e.g. the interference of bilirubin with iron chelation.

In a preferred embodiment, the methods and apparatus of this invention provide a highly accurate means for determining total bilirubin with a reagent composition which includes a diazonium salt (or what is also known as a diazo reagent).

Any of a great number of diazo reagents can be used in this invention although some, because of their instability in dry form, may be limited in utility to solution or "wet" assay. Examples of useful diazo reagents include 2,6-dichlorobenzene diazonium salts and the like as described, for example, in U.S. Patent 3,880,588 (issued April 29, 1975 to Rittersdorf et al), 2,4-dichlorobenzenediazonium salt and the like as described in U.S. Patent 4,038,031 (issued July 26, 1977 to Lam), diazotized sulfanilic acid, diazotized 2,4-dichloroaniline, diazonium fluoroborate, and others known in the art.

Particularly useful diazonium salts are those described in U. S. Patent 4,468,467, noted above. Those salts have the advantage of being extremely resistant to shock induced decomposition and therefore, can be used for both solution and dry assays. These diazonium salts have the structure:



wherein X^- is a stabilizing anion and Y is -CO- or $-SO_2-$.

R^1 and R^2 are independently selected from hydrogen, substituted or unsubstituted alkyl, preferably having from 1 to 20 carbon atoms (e.g. methyl, chloromethyl, isopropyl, dodecyl), substituted or unsubstituted aralkyl, preferably having from 7 to 20 carbon atoms in the aralkyl backbone (e.g. benzyl), substituted or unsubstituted aryl, preferably having from 6 to 14 carbon atoms in the aromatic backbone (e.g. phenyl, xylyl, p-methoxyphenyl, naphthyl), and carboxyalkyl and hydroxyalkyl, preferably wherein the alkyl group is lower alkyl, i.e. having 1 to 4 carbon atoms [e.g. carboxymethyl, carboxyethyl, hydroxymethyl, hydroxyethyl, tris-(hydroxymethyl)methyl and hydroxy-4-n-butyl], and more preferably are not both hydrogen.

R^3 and R^4 are independently selected from groups which are electron donor groups or mildly electron withdrawing groups, such that the sum of the Hammett sigma values for R^3 and R^4 does not exceed +0.4. Examples of such groups include hydrogen, halogen (e.g. chloro, bromo), lower alkyl preferably of 1 to 4 carbon atoms (e.g. methyl, propyl), alkylthio preferably of 1 to 4 carbon atoms (e.g. methylthio), lower alkoxy preferably of 1 to 4 carbon atoms (e.g. methoxy, ethoxy), aralkoxy preferably of 1 to 10 carbon atoms in the aralkoxy backbone (e.g. benzyloxy), phenylthio, and alkylamino preferably of 1 to 8 carbon atoms (e.g. acetamino). Alternatively, R^3 and R^4, taken together, represent the carbon atoms necessary to complete a fused carbocyclic arylene moiety, such as naphthylene, indylene, or anthrylene, including such ring structures substituted with the other groups identified for R^3 and R^4.

Stabilizing anions for these diazonium salts are known. These anions make possible the isolation of the salts in dry form and provide for long term thermal stability as well as reduced shock sensitivity. In the formula above, X^- is preferably the anion of a Lewis acid coordinatively saturated by a hydrogen halide. Useful stabilizing anions include tetrafluoroborate, hexafluorophosphate, chlorozincate and hexafluorotitanate. Hexafluorophosphate has been found to be particularly preferred. Other useful anions include arylsulfonates, such as naphthylene disulfonate and 4,4'-biphenyldisulfonate.

Useful diazonium salts include:

4-(N-carboxymethylsulfamyl)benzene diazonium hexafluorophosphate,

4-[N,N-bis(carboxymethyl)sulfamyl]benzenediazonium hexafluorophosphate,

4-[N,N-bis(2-hydroxyethyl)sulfamyl]benzenediazonium hexafluorophosphate,

4-(N-carboxymethylcarbamyl)benzenediazonium tetrafluoroborate,

4-(N-carboxypropylcarbamyl)benzenediazonium naphthylenedisulfonate,

4-(N-carboxymethylsulfamyl)benzenediazonium tetrafluoroborate,

4-(N-dodecylsulfamyl)benzenediazonium tetrafluoroborate,

3,5-dichloro-4-(N-carboxymethylsulfamyl)-benzenediazonium hexafluorophosphate,

4-(N-carboxymethylsulfamyl)-1-diazonium naphthylene hexafluorophosphate,

7-[N-tris(hydroxymethyl)methylcarbamyl]-4-diazoniumindene hexafluorophosphate, and

4-[N,N-bis(carboxymethyl)sulfamyl]-1-diazonium-6-methoxy naphthylene chlorozincate.

The interactive compositions useful in the determination of total bilirubin can include an acid. Where the composition is in the form of an aqueous solution, any acid is useful including mineral acids, such as hydrochloric and sulfuric acids. Where a dry reagent composition is desired, acids which are solid when anhydrous can be used. Useful acids of this type include malic, sulfosalicylic, tartaric, succinic, phthalic, cyclohexanesulfamic, p-toluenesulfonic and citric. Other useful acids, preformed or formed in situ during the assay, are known to one skilled in clinical chemistry.

The amount of acid used varies widely. Generally, the amount of acid is sufficient to maintain the pH of the reagent composition between 1 and 7 when contacted with water. In a preferred embodiment, the acid used is 3,3-dimethylglutaric acid (or equivalent alkali metal salt) which is present in an amount effective to maintain the pH at 3.5 or less when contacted with water.

The bilirubin-determining interactive composition can also include what is known in the art as a "diazo bilirubin promoter" (sometimes also referred to as an "accelerating agent"). Useful promoters include dyphylline, caffeine, sodium acetate, sodium benzoate and gum arabic.

The present invention can be practiced with any chemical analyzer constructed to perform rate or endpoint colorimetric assays. Such analyzers can also have the capability of performing potentiometric assays.

Response measuring means measure the spectrophotometric response in analytical elements. Generally, such a spectrophotometric response is a spectral absorption which can be quantified by measuring the transmission or reflection density in the element with a suitable spectrophotometer containing a light source,

photodetector and one or more filters. Such filters are placed, if desired, either between the light source and the element or between the element and the photodetector.

Referring to FIG. 1, the signal generated by photodetector 90 is directed, via amplifier 92 and A/D converter 94, to computing means 100 which functions with a central processing unit 110 to control the operations and calculations of the analyzer. Such computing means can be directly attached to the analyzer or be an off-line component, and includes memory units 120 as known in the art. It can further include input/output (I/O) devices, such as keyboard 130 and display 140. Computing means 100 also includes driver interface boards (not shown) to convert computer signals to signals that control the motors of the various moving components of the analyzer.

A variety of conventional computing means (e.g. computer or programmable microprocessor) are useful in supplying the above-noted features. Such computing means of the analyzer calculates the analyte concentration or activity in the test sample using the equation (I):

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n]$$

wherein C is analyte concentration or activity, $A_1, A_2, \cdots A_n$ are the spectrophotometric responses measured at $\lambda_1, \lambda_2, \cdots \lambda_n$, respectively, and a_0, a_1 and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ are constants determined by the calibration method of this invention described below. The wavelengths $\lambda_1, \lambda_2, \cdots \lambda_n$ are determined as described below. The concentration of an analyte can be determined with an endpoint assay, and the activity of an analyte (e.g. an enzyme) can be determined from a rate assay.

The analyzer can be of any type which has the capability of determining analytes such as by colorimetric, radiometric, fluorometric or

potentiometric means and of the type which is capable of doing either endpoint or rate assays. Chemical analyzers which can be adapted for use with the present invention include those described in U.S. Patents 4,152,390 (issued May 1, 1979 to Nosco et al), 4,224,032 (issued September 23, 1980 to Glover et al), 4,287,155 (issued September 1, 1981 to Tersteeg et al), and 4,420,566 (issued December 13, 1983 to Jessop et al). Particularly useful chemical analyzers include the EKTACHEM 400 and 700 analyzers available from Eastman Kodak Company (Rochester, N.Y.).

A chemical analyzer is adapted, or calibrated, to determine an analyte in an aqueous liquid as described herein by the following procedure. Basically, the analyzer must be calibrated by recording therein in some manner the a_0, a_1, and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ constants which are used in equation (I) in analyte determination:

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n].$$

In determining these constants, a calibrator curve is prepared using samples of known analyte concentration and a suitable reference analytical method. For instance, in the illustrated example below for total bilirubin, a calibrator curve is prepared using a modified Jendrassik-Grof reference method [see e.g. Doumas et al, Clin. Chem., 19, pp. 984-993 (1973)] at a wavelength of 600 nm.

A multiplicity m (e.g. 100) of patient test samples (i.e. samples obtained from a random population of patients) are assayed for analyte concentration or activity C_j, where j=1 to m, using the reference method. The number of patient test samples is empirically chosen and can vary widely depending upon the number of samples needed to clearly show the effect of an undefined interferent. Concentrations C_j are determined from the calibration curve. These same patient test samples are then assayed using a conventional assay

(e.g. the assay of U.S. Patent 4,468,467, noted above for total bilirubin determination). Test results which show a significant bias (positive or negative) relative to the reference method on a methods comparison plot indicate test samples which exhibit effects of the undefined interferent during the assay. As used herein, the term "significant" refers to a % bias of greater than 50%. Bias is a term used to describe the difference between a test value observed with a reference method and the test value observed with the conventional assay. Any of a number of conventional methods are known in the art for a given analyte.

From the multiplicity of patient test samples assayed and plotted as described above, a first patient test sample ("biased" sample) is identified which exhibits the significant bias, and a second patient test sample ("unbiased" sample) is identified which does not exhibit the significant bias. A spectral absorption scan is then made of each of these patient test samples over the entire range of the visible spectrum. The procedure and equipment used for making such scans are well known in the art.

Absorption bands are then determined from these spectral absorption scans, by plotting a spectral absorption difference scan. Such a scan is a plot of the difference of the two spectral absorption scans obtained by subtracting the scan of the second patient test sample from the scan of the first patent test sample. The absorption difference spectrum characterizes the difference in spectral response between the "biased" test sample and the "unbiased" test sample. Absorption difference spectra and the procedure and equipment used to prepare them are known in the art.

The absorption difference spectrum so prepared will have two or more absorption bands or regions of peak difference up to n absorption bands, including that around primary wavelength λ_1. Generally, the primary wavelength λ_1 is chosen as the conventional wavelength at which a given analyte is spectrophotometrically measured. In each absorption band other than the absorption band around λ_1 is a wavelength which is representative of that band and can be used to correct the test result for interferent effects. At least one of those secondary wavelengths, $\lambda_2, \lambda_3, \cdots \lambda_n$ is chosen for use in the succeeding steps. In the bilirubin example below, a single secondary wavelength, λ_2=460 nm was chosen within the absorption band seen in the difference spectrum other than the absorption band at λ_1=540 nm.

Next, using a multiplicity (e.g. at least 100) of patient test samples of known analyte concentrations or activities, a regression line is determined and its intercept and slopes are calculated using multiple linear regression analysis and the equation (II):

$$C = a_0 + a_1A_1 + a_2A_2 + \cdots + a_nA_n$$

wherein C is the known analyte concentration or activity, $A_1, A_2, \cdots A_n$ are the spectrophotometric responses measured with those test samples at $\lambda_1, \lambda_2, \cdots \lambda_n$, respectively, and a_0 is the intercept and $a_1, a_2, \cdots a_n$ are the slopes to be determined. For total bilirubin (B_T), equation II becomes IIa:

$$B_T = a_0 + a_1A_1 + a_2A_2$$

wherein A_1 and A_2 are the spectrophotometric responses measured at λ_1 and λ_2, respectively.

The spectrophotometric responses $A_1, A_2, \cdots A_n$ are either spectral absorbance (in the case of solution assays) or reflection density

(D_R). Where D_R is not linear with analyte concentration or activity, it is converted to transformed transmission density (D_T) where D_T is linear with analyte concentration or activity. The analyzer can be programmed to convert D_R to D_T using what are known in the art as transformation equations. Particularly useful transformation equations are known as Clapper-Williams Transforms which are described by Williams et al in J. Opt. Soc. Am., 43, 595(1953).

Once the constants $a_0, a_1, a_2, \cdots a_n$ are known from the linear regression analysis, the constants $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ used in equation (I) noted above can be computed using the equation (III):

$$\alpha_i = \frac{a_{i+1}}{a_1} \text{ wherein } i = 1 \text{ to } (n-1).$$

The constants a_0, a_1 and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ so determined are then recorded or stored in the analyzer for use during analyte determination when equation (I) is solved for unknown analyte concentration or activity C. These constants can be stored, for example, in computing means 100 (FIG. 1), or on a "soft-copy" of the program which is separately inserted into the analyzer during use. The analyzer can be calibrated as often as desired, for example, as part of routine weekly analyzer calibration procedures or when new lots of reagents, test solutions or analytical elements are used.

FIG.2 represents a logic flow chart that is useful in programming a microprocessor to accomplish the described calibration method. From this flow chart, a program routine is readily determinable using conventional programming techniques.

The analyte determination method of this invention is adaptable to both solution (i.e. "wet chemistry") and dry element (i.e. "dry chemistry") assays. In solution assays, the assay is carried out

entirely in a liquid medium by mixing an aqueous sample to be assayed with a solution containing the interactive composition. The resulting mixture is incubated at an appropriate temperature if desired. This solution assay technique is well known in the art.

When the method is employed with "dry chemistry" elements, the interactive composition can be incorporated into a suitable absorbent carrier matrix by imbibition, impregnation, coating or another suitable technique. Useful carrier matrices are insoluble and maintain their structural integrity when exposed to water or physiological fluids such as urine or serum. Useful carrier matrices can be prepared from porous materials such as paper, cellulose, porous particulate structures, wood, glass fiber, woven and nonwoven fabrics (synthetic and nonsynthetic) and the like. A useful dry analytical element is made by imbibing a solution of the reagent composition into the matrix and drying. Useful materials and procedures for making such elements are well known in the art as exemplified in U.S. Patents 3,092,465 (issued June 4, 1963 to Adams et al), 3,802,842 (issued April 9, 1974 to Lange et al), 3,915,647 (issued October 28, 1975 to Wright), 3,917,453 (issued November 4, 1975 to Milligan et al), 3,936,357 (issued February 3, 1976 to Milligan et al), 4,248,829 (issued February 3, 1981 to Kitajima et al), 4,255,384 (issued March 10, 1981 to Kitajima et al), and 4,270,920 (issued June 2, 1981 to Kondo et al), U.K. Patent 2,052,057 (published January 21, 1981), and U.S. Patent 4,468,467, noted above, as well as the patents noted below.

In dry element bilirubin assays, the diazonium salt is generally present at a coverage of at least 0.05 g/m^2.

Preferably, the analytical elements useful in the assay of this invention have at least one porous spreading zone (e.g. which can also be a spreading/reagent zone containing the interactive composition), which is preferably isotropically porous. This zone can be a self-supporting carrier matrix (i.e. composed of a material rigid enough to maintain its integrity) or carried on a separate support. A support is a substrate made of any suitable dimensionally stable, and optionally, transparent (i.e. radiation transmissive) material which transmits electromagnetic radiation of a wavelength between 200 and 900 nm. Useful support materials include polystyrene, polyesters [e.g. poly(ethylene terephthalate)], polycarbonates, cellulose esters, etc. The element can have a plurality of zones (spreading, spreading/reagent, reagent, subbing, hydrophilic, mordant, buffer, etc.), some or all containing reagents. These zones are in fluid contact with each other, meaning that fluids can pass between superposed regions of adjacent zones. The zones can be separate coated layers, although one or more zones can be in a single layer, or one or more separate layers can be in a single zone of an element. Dry element formats and materials are known in the art and described, for example, in U.S. Patents 3,992,158 (issued November 16, 1976 to Przybylowicz et al), 4,042,335 (issued August 16, 1977 to Clément), 4,144,306 (issued March 13, 1979 to Figueras), 4,132,528 (issued January 2, 1979 to Eikenberry et al), and 4,258,001 (issued March 24, 1981 to Pierce et al).

The porous spreading zone is generally a layer which can accept (i.e. absorb completely) an aqueous liquid sample of at least 1 µℓ. When the sample is applied directly to the zone or provided to it from a zone or zones in fluid contact with it, the

sample is distributed such that a uniform concentration of the sample is provided at the surface of the spreading zone facing an adjacent zone. Useful materials for preparing spreading zones are described, for example, in U.S. Patents 3,992,158 and 4,258,001, noted above, and 4,292,272 (issued September 29, 1981 to Kitajima et al), West German OLS 3,150,102 (published July 29, 1982), and Japanese Patent Publication 57(1982)-101760 (published June 24, 1982). The spreading zone, for example, can be composed of either fibrous or non-fibrous materials, or both.

A variety of different elements can be prepared and used in accordance with the present invention. Elements can be configured in a variety of forms, including elongated tapes of any desired width, sheets or chips.

The analyte determination method of this invention can be manual or automated. For example, the amount of analyte (e.g. total bilirubin) in an aqueous liquid is determined by taking an element from a supply roll, slide packet or other source and physically contacting it with a sample of the liquid, e.g. in a suitable chemical analyzer. Such contact can be accomplished in any suitable manner, e.g. dipping or immersing the element into the sample or, preferably, by spotting the element by hand or machine with a drop (e.g. 1-20 μl) of the sample by pipette or another suitable dispensing means.

After sample application, the element is exposed to any conditioning, such as incubation, heating or the like, that may be desirable to quicken or otherwise facilitate obtaining any test result.

The analyte (e.g. bilirubin), if present, then reacts with the interactive composition and produces a detectable response or signal, which

response is quantifiable by passing the element through a zone in which suitable apparatus for spectrophotometric detection (e.g. reflection or transmissive spectrophotometry) is provided.

The spectrophotometric responses are determined at a multiplicity (i.e. two or more) of absorption wavelengths as described above with regard to the calibration method of this invention. The exact wavelengths chosen to measure absorption are determined by that method and depend upon the analyte and choice of reagents. For example, depending upon the diazonium reagents used for bilirubin determination, the primary wavelength (λ_1) is within the range of from 500 to 580 nm. A secondary wavelength (λ_2) for total bilirubin is within the range of from 420 to 490 nm.

The concentration or activity of the analyte, e.g. total bilirubin, is then determined according to equation (I) noted above.

The following example is presented to illustrate the practice of this invention. In this example, the sources of materials were as follows: polyurethane resin as Estane 5715 from B. F. Goodrich Co. (Cleveland, Ohio), dyphylline from Aldrich Chemicals Co. (Milwaukee, Wisconsin), Triton X-100 surfactant from Rohm & Haas (Philadelphia, Pennsylvania), Surfactant 10G surfactant from Olin Mathieson Corp. (Stamford, Connecticut), and the remainder from Eastman Organic Chemicals (Rochester, New York).

Example - Total Bilirubin Assay Using Dry Analytical Element

This example illustrates the present invention as practiced in determining total bilirubin in human sera.

A dry analytical element having the format and components shown below was prepared and used to determine total bilirubin at λ_1=540 nm according to the teaching of U.S. Patent 4,468,467, noted above, in a population of human serum samples. In approximately 10% of the serum samples, the predicted total bilirubin values were positively biased from 100 to 300% as compared to predicted values determined by the modified Jendrassik-Grof reference method noted above. It was noted that a majority of the samples giving biased predictions were from hemodialysis or other renal-defective patients. Attempts to isolate the interferent were unsuccessful. Hence, it is believed that the interferent is formed *in situ*, i.e. when the sample is contacted with the diazonium salt during the assay.

Element Format:

Spreading/ Reagent Layer	Barium sulfate	110 g/m²
	Cellulose acetate	9 g/m²
	Polyurethane resin	5.5 g/m²
	Dyphylline	2.2 g/m²
	Triton X-100 surfactant	g/m²
	4-(N-carboxymethylsulfamyl)benzenediazonium hexafluorophosphate	1 g/m²
Subbing Layer	Poly(N-isopropylacrylamide)	0.4 g/m²
Hydrophilic/ Layer	Gelatin (hardened)	9 g/m²
	Malic acid (pH 5)	2.9 g/m²
	Poly(styrene-*co*-N-vinylbenzyl-N-benzyl-N,N-dimethylammonium-chloride-*co*-divinylbenzene)	1.7 g/m²
	Surfactant 10G surfactant	0.1 g/m²
Support	Poly(ethylene terephthalate)	

The same dry element was used in the practice of this invention and it was found that the positive bias observed with the known assay was substantially eliminated with the present invention.

The improved assay of this invention was accomplished with an EKTACHEM 400 clinical chemistry analyzer which had been calibrated to determine total bilirubin (B_T) concentration according to the equation (IV):

$$B_T = a_0 + a_1[A_1 + \alpha_1 A_2]$$

which is equation (I) shown above wherein n is 2. The calibration method of this invention was used to determine the constants a_0, a_1 and α_1 and to record them in the microprocessor of the analyzer.

In particular, the calibration method of this invention was carried out by taking 1615 random population human serum samples and identifying in those samples a first sample which exhibited a significant positive bias with respect to a bilirubin calibration curve generated using the reference method noted above, and second sample not having a bias, but both samples having essentially identical total bilirubin concentration of about 1.5 mg/dℓ as determined by the reference method. The total bilirubin concentration was evaluated at a primary wavelength λ_1=540 nm which is the conventional absorption wavelength for bilirubin determinations using the assay of U.S. Patent 4,468,467 noted above. This assay was the conventional assay used in this example.

Spectral scans were plotted for the two identified samples. From a spectral absorption difference scan determined by subtracting the spectral scan of the second sample from the spectral scan of the first sample, it was observed that those scans were quite different over an absorption band

centering at 460 nm. It was thus decided to select 460 nm as a secondary wavelength (λ_2) for measuring A_2 in the above equation (IV) in order to correct for the observed effect of the interferent in the first sample.

Using another random population of 1100 human serum samples of known bilirubin concentration, a linear regression analysis was performed on equation (IIa) $B_T=a_0+a_1A_1+a_2A_2$, wherein A_1 and A_2 are the transformed transmission densities (D_T) observed at λ_1=540 nm and λ_2=460 nm, respectively. The reflection densities (D_R) were converted to transformed transmission densities (D_T) using the Clapper-Williams Transforms described above. The constants a_0, a_1 and a_2 were then determined from the regression analysis. The constant a_0, which is the intercept of the linear regression line was determined to be -1.5, a_1 which is the slope of the line with respect to A_1 was determined to be 162.5, and a_2 which is the slope of the line with respect to A_2 was determined to be -24.25. Equation IIa was then modified to be (IIb):

$$B_T = -1.5 + 162.5\ A_1 - 24.25\ A_2.$$

Dividing the last two terms by a_1 (i.e. 162.5) gives equation (IIc):

$$B_T = 1.5 + 162.5\ [A_1 - 0.15\ A_2]$$

wherein α_1=-0.15. The values of these constants were than programmed into the EKTACHEM 400 analyzer for use in total bilirubin assays.

These assays were performed by feeding dry analytical elements having the format noted above into the calibrated EKTACHEM 400 analyzer and contacting the elements with a 10 µℓ sample of each of the 1100 human serum samples noted above. The

analyzer calculated the total bilirubin concentrations (mg/dl) according to equation (IIc) by measuring D_R and transforming it into D_T. The same serum samples were also evaluated for total bilirubin using the assay of U.S. Patent 4,468,467, noted above, using an EKTACHEM 400 chemical analyzer which had not been calibrated according to this invention.

Table I presented below lists the data found in this comparison of the two assays. These data are determined from a methods comparison plot which plots the total bilirubin concentration of the method of this invention against the total bilirubin concentration of the conventional method. It is appparent from the data that the assay of this invention reduces the bias observed with the known assay and significantly improves the accuracy of total bilirubin determination.

In particular, the Sy•x value for Example 1 was significantly lower than the corresponding Control value. The Sy•x value is a conventional statistic which is a measure of the scatter of data points about the regression line. The lower this value is, the more precise the assay. The r value is a conventional statistic describing the degree of association between the two methods. The closer this value is to 1.0, the more accurate is the assay. The present invention had a r value significantly closer to 1.0 than did the Control assay.

Table I

	Control	Example 1
Sy•x	0.498	0.309
Correlation coefficient(r)	0.949	0.981

CLAIMS:

1. A method for calibrating a chemical analyzer useful in the determination of an analyte in an aqueous liquid, the analyzer comprising a) spectrophotometric means for detecting "n" spectrophotometric responses $A_1, A_2, \cdots A_n$ resulting when a sample of the liquid is contacted with an interactive composition for the analyte, and b) means for calculating the concentration or activity C of the analyte in the sample using the equation (I):

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n]$$

wherein $a_0, a_1, \alpha_1, \alpha_2, \cdots \alpha_{n-1}$ are defined below, said method characterized by the steps of:

A. from a multiplicity of patient test samples of unknown analyte concentration or activity, identifying first and second patient test samples having substantially the same analyte concentration or activity, the first sample exhibiting a significant bias in analyte concentration or activity and the second sample exhibiting no significant bias in analyte concentration or activity measured at a primary wavelength λ_1,

B. making a spectral absorption scan of each of the samples identified in step A,

C. identifying absorption bands from the spectral scans where differences in absorbance between the scans can be observed, and selecting at least one secondary wavelength from the group of secondary wavelengths $\lambda_2, \lambda_3, \cdots \lambda_n$ representative of the absorption bands of both of the first and second patient samples, respectively, wherein n represents the number of absorption bands,

D. using a multiplicity of patient test samples of known analyte concentration or activity, determining a linear regression line and its intercept and slopes using the equation (II):

$$C = a_0+a_1A_1+a_2A_2+\cdots+a_nA_n$$

wherein C is analyte concentration or activity; a_0 is the intercept of the line, $A_1, A_2, \cdots A_n$ are the spectrophotometric responses measured at $\lambda_1, \lambda_2, \cdots \lambda_n$ respectively, and $a_1, a_2, \cdots a_n$ are the slopes of the line relating the spectrophotometric responses at $\lambda_1, \lambda_2, \cdots \lambda_n$, respectively, to the analyte concentration or activity,

E. using the results of step D to determine constants $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ for equation (I) above using the equation (III):

$$\alpha_i = \frac{a_{i+1}}{a_1}$$ wherein i = 1 to (n-1), and

F. recording in the analyzer the values of the constants a_0, a_1, and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ for use in equation I above.

2. The calibration method as claimed in claim 1 wherein the absorption bands are identified in step C by subtracting the spectral scan of the second patient test sample from the spectral scan of the first patient test sample, and making a spectral absorption difference scan.

3. A method for the determination of an analyte in an aqueous liquid, the method comprising physically contacting a sample of the liquid with an interactive composition for the analyte to generate a spectrophotometric response, and measuring the spectrophotometric response,

the method characterized by measuring the spectrophotometric responses $A_1, A_2, \cdots A_n$ resulting from the contact at, respectively, a primary wavelength λ_1 and at n secondary wavelengths selected from secondary wavelengths $\lambda_2, \lambda_3, \cdots \lambda_n$ determined according to the calibration method claimed in either claim 1 or 2, and determining the concentration or activity C of the analyte using the equation (I):

$$C = a_0+a_1[A_1+\alpha_1A_2+\cdots+\alpha_{n-1}A_n]$$

wherein the constants a_0, a_1 and α_1, α_2, $\cdots\alpha_{n-1}$ are determined according to the calibration method claimed in either claim 1 or 2, for as many n secondary wavelengths as are used.

4. The determination method as claimed in claim 3 wherein the interactive composition is in a dry analytical element which comprises a support having thereon an isotropically porous spreading zone.

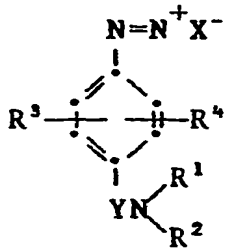
5. The determination method as claimed in either claim 3 or 4 wherein the analyte is bilirubin.

6. The determination method as claimed in claim 5 wherein n is 2 and the concentration of total bilirubin (B_T) in the liquid is determined using the equation:

$B_T = a_0+a_1[A_1+\alpha_1A_2]$ wherein a_0, a_1 and α_1 are constants determined according to the calibration method claimed in either claim 1 or 2, and A_1 and A_2 are spectrophotometric responses detected at λ_1 and λ_2, respectively.

7. The determination method as claimed in any one of claims 3 to 6 wherein λ_1 is from 500 to 580 nm and λ_2 is from 420 to 490 nm.

8. The determination method as claimed in any one of claims 5 to 7 wherein the interactive composition comprises a diazonium salt which is represented by the structure:



wherein X^- is a stabilizing anion, Y is -CO- or $-SO_2-$, R^1 and R^2 are independently selected from hydrogen, alkyl, aralkyl, aryl, carboxyalkyl and

hydroxyalkyl, and R^3 and R^4 are independently selected from groups such that the sum of the Hammett sigma values for R^3 and R^4 does not exceed +0.4 or R^3 and R^4, taken together, represent the carbon atoms necessary to complete a fused carbocyclic arylene moiety.

9. The invention as claimed in any one of claims 1 to 8 wherein the spectrophotometric responses $A_1, A_2, \cdots A_n$ are transformed transmission densities (D_T) determined at $\lambda_1, \lambda_2, \cdots \lambda_n$, respectively.

10. A chemical analyzer for the determination of an analyte in an aqueous liquid in contact with an interactive composition for the analyte,

the analyzer characterized by comprising

means for measuring the spectrophotometric responses $A_1, A_2, \cdots A_n$ at, respectively, a primary wavelength λ_1 and at n secondary wavelengths selected from the secondary wavelengths $\lambda_2, \lambda_3, \cdots \lambda_n$, and

means for determining the concentration or activity C of the analyte using the equation (I):

$$C = a_0 + a_1[A_1 + \alpha_1 A_2 + \cdots + \alpha_{n-1} A_n]$$

wherein the constants a_0, a_1 and $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ are determined according to the calibration method as claimed in either claim 1 or 2, for as many n secondary wavelengths as are used.

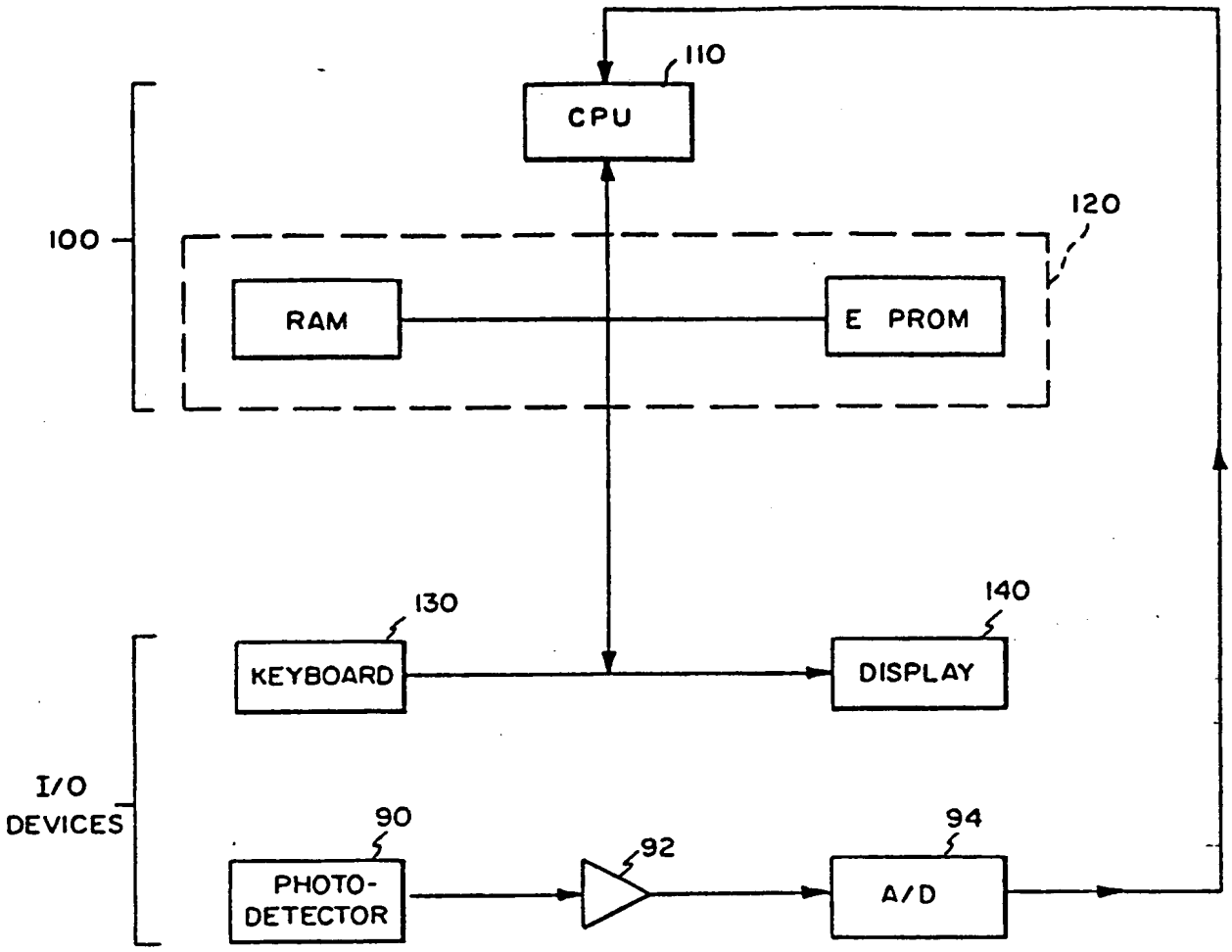

110
CPU
100
120
RAM
E PROM
130
140
KEYBOARD
DISPLAY
I/O
DEVICES
90
92
94
PHOTO-
DETECTOR
A/D

FIG. 1

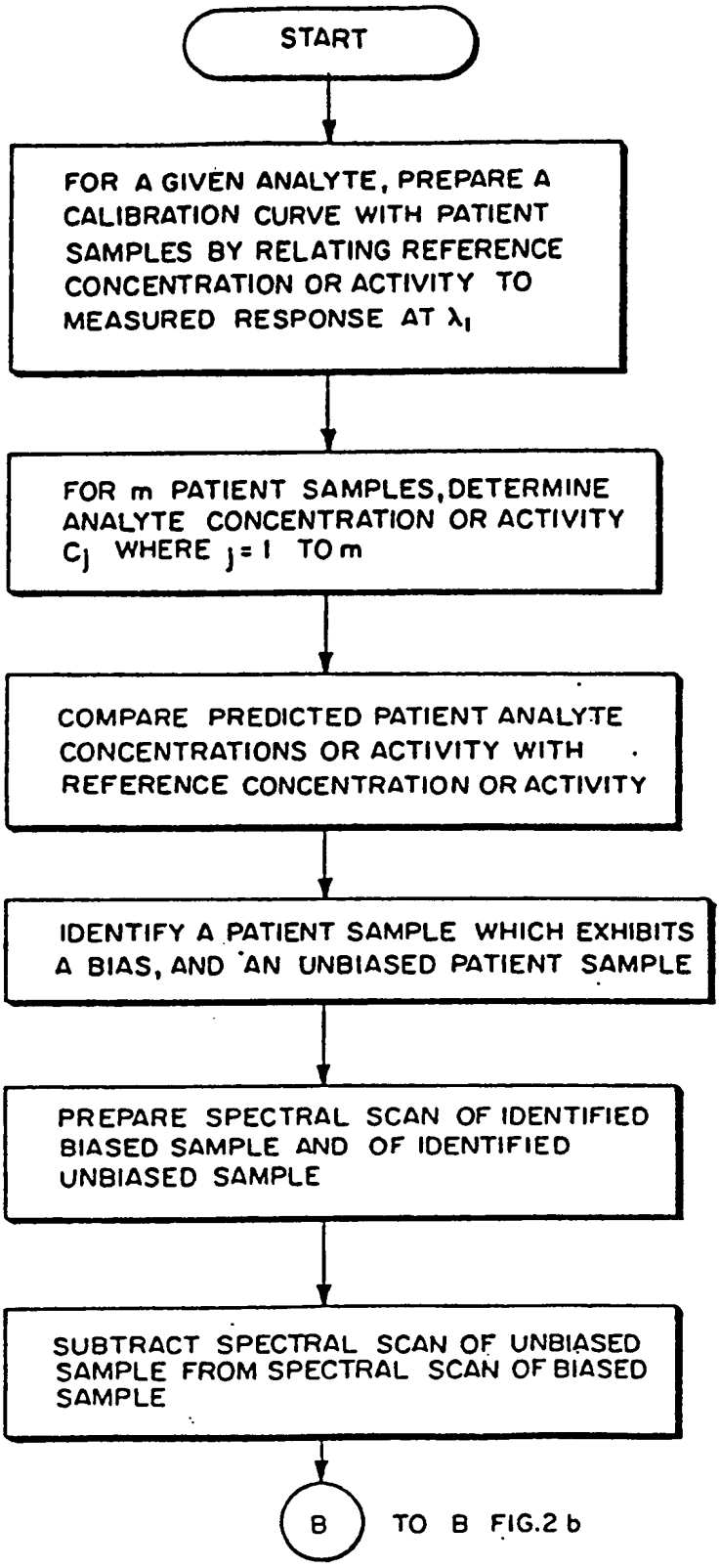

START
FOR A GIVEN ANALYTE, PREPARE A CALIBRATION CURVE WITH PATIENT SAMPLES BY RELATING REFERENCE CONCENTRATION OR ACTIVITY TO MEASURED RESPONSE AT λ_1
FOR m PATIENT SAMPLES, DETERMINE ANALYTE CONCENTRATION OR ACTIVITY C_j WHERE $j = 1$ TO m
COMPARE PREDICTED PATIENT ANALYTE CONCENTRATIONS OR ACTIVITY WITH REFERENCE CONCENTRATION OR ACTIVITY
IDENTIFY A PATIENT SAMPLE WHICH EXHIBITS A BIAS, AND AN UNBIASED PATIENT SAMPLE
PREPARE SPECTRAL SCAN OF IDENTIFIED BIASED SAMPLE AND OF IDENTIFIED UNBIASED SAMPLE
SUBTRACT SPECTRAL SCAN OF UNBIASED SAMPLE FROM SPECTRAL SCAN OF BIASED SAMPLE
B
TO B FIG.2 b

FIG.2 a

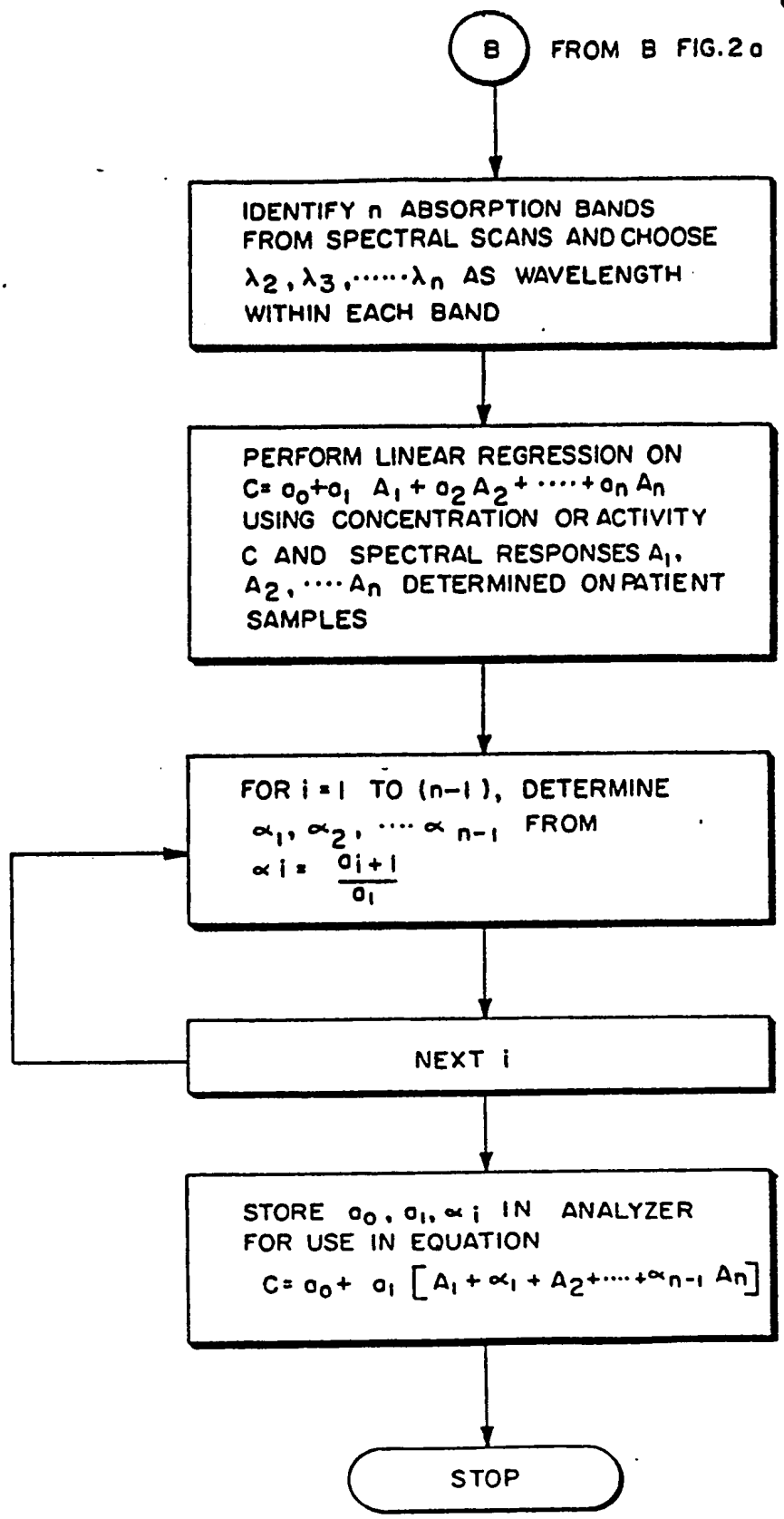

B
FROM B FIG.2a
IDENTIFY n ABSORPTION BANDS FROM SPECTRAL SCANS AND CHOOSE $\lambda_2, \lambda_3, \cdots \lambda_n$ AS WAVELENGTH WITHIN EACH BAND
PERFORM LINEAR REGRESSION ON $C = a_0 + a_1 A_1 + a_2 A_2 + \cdots + a_n A_n$ USING CONCENTRATION OR ACTIVITY C AND SPECTRAL RESPONSES $A_1, A_2, \cdots A_n$ DETERMINED ON PATIENT SAMPLES
FOR i = 1 TO (n-1), DETERMINE $\alpha_1, \alpha_2, \cdots \alpha_{n-1}$ FROM $\alpha i = \frac{a_{i+1}}{a_1}$
NEXT i
STORE $a_0, a_1, \alpha i$ IN ANALYZER FOR USE IN EQUATION $C = a_0 + a_1 [A_1 + \alpha_1 + A_2 + \cdots + \alpha_{n-1} A_n]$
STOP

FIG. 2 b